Exhibit B

Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-$APP_{Sw,Ind}$) mice

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Neurogenesis continues in the adult brain and is increased in certain pathological states. We reported recently that neurogenesis is enhanced in hippocampus of patients with Alzheimer's disease (AD). We now report that the effect of AD on neurogenesis can be reproduced in a transgenic mouse model. PDGF-$APP_{Sw,Ind}$ mice, which express the Swedish and Indiana amyloid precursor protein mutations, show increased incorporation of BrdUrd and expression of immature neuronal markers in two neuroproliferative regions: the dentate gyrus and subventricular zone. These changes, consisting of ≈2-fold increases in the number of BrdUrd-labeled cells, were observed at age 3 months, when neuronal loss and amyloid deposition are not detected. Because enhanced neurogenesis occurs in both AD and an animal model of AD, it seems to be caused by the disease itself and not by confounding clinical factors. As neurogenesis is increased in PDGF-$APP_{Sw,Ind}$ mice in the absence of neuronal loss, it must be triggered by more subtle disease manifestations, such as impaired neurotransmission. Enhanced neurogenesis in AD and animal models of AD suggests that neurogenesis may be a compensatory response and that measures to enhance neurogenesis further could have therapeutic potential.

Neurogenesis occurs in the adult brain and can be stimulated further by pathological processes, suggesting that newly generated neurons might be capable of replacing cells that are lost in neurological diseases. Animal models have been useful in identifying and characterizing injury-induced neurogenesis associated with epilepsy (1), ischemic stroke (2), and Parkinson's disease (3). Neurogenesis triggered by ischemia in rodents, for example, is associated with migration of newborn neurons from their sites of origin in the subventricular zone (SVZ) or dentate gyrus subgranular zone (DG-SGZ) into injured areas of the brain (4–6). Neurogenesis also generates functional neurons in adult human brain (7), and increased neurogenesis has been reported in patients with Huntington's disease (8) and Alzheimer's disease (AD) (9). These findings are encouraging with respect to prospects for cell-replacement therapy because the persistent stimulus-responsiveness of neurogenesis in neurodegenerative diseases indicates that additional stimulation and regulation by therapeutic interventions may be possible.

Recently, we found that neurogenesis is increased in the DG-SGZ from patients with AD (9). Compared to controls, AD brains showed increased expression of the immature neuronal markers doublecortin (DCX), embryonic nerve cell adhesion molecule, neurogenic differentiation factor Neuro D, and turned-on-after-division/Ulip-1/CRMP-4. Expression of DCX and turned-on-after-division/Ulip-1/CRMP-4 was associated with neurons in DG-SGZ, the DG granule cell layer, which is the physiological destination of these neurons, and the CA1 region of Ammon's horn, which is the principal site of hippocampal pathology in AD. These findings suggest that neurogenesis is increased in AD hippocampus, where it could give rise to cells that replace lost neurons, and that stimulating hippocampal neurogenesis could provide a unique approach to AD treatment.

Animal models are critical for evaluating and optimizing prospective therapies, including cell-replacement therapy for neurological diseases. The ability to recapitulate clinical features in an animal model also helps to establish whether such features are inherent to the disease or merely associations related, for example, to nutritional state or medications. Several mouse models of AD have been developed, most of which involve transgenic expression of one or more mutations found in familial AD, but none of which reproduces the full spectrum of changes seen in the human disease (10–12).

The pathophysiology of AD has been studied extensively in transgenic models, but neurogenesis in AD models has received little attention. In related studies, 11- to 14-month-old transgenic mice that express amyloid precursor protein (APP) with the Swedish [APP695(K595N/M596L)] mutation showed reduced numbers of BrdUrd-, embryonic neural cell adhesion molecule-, and BrdUrd/embryonic neural cell adhesion molecule-labeled cells in DG or SVZ, consistent with impaired neurogenesis (13, 14). In another study, 24-month-old APP23 [APP751(K670N/M671L)] mice (15) showed a large increase in BrdUrd labeling in cerebral neocortex, but BrdUrd-immunopositive cells were NeuN-immunonegative (16), and neither DG nor SVZ was studied. Presenilin 1 (PS1), which is mutated in some cases of familial AD, has also been implicated in neurogenesis, in that increased expression of WT, but not familial AD mutant, PS1 increases hippocampal neurogenesis (17), and environmental enrichment-induced (but not basal) neurogenesis is impaired in the DG-SGZ of PS1-knockout mice (18).

In light of our finding of increased neurogenesis in AD and the inconclusive findings from animal studies, we investigated neurogenesis in DG-SGZ and in SVZ of platelet-derived growth factor-β (PDGF)-$APP_{Sw,Ind}$ transgenic mice (19), which express human APP isoforms APP695, APP751, and APP770 with the Indiana (V717F) and Swedish (K670N/M671L) mutations, driven by a PDGF promoter. Each of these is a missense mutation that arises from a single base change and, in humans, produces autosomal dominant, early-onset AD, which becomes symptomatic at 45–55 years of age and leads to death within ≈7 years. $APP_{Sw,Ind}$ mice exhibit extracellular deposits of β-amyloid that can be detected beginning at 6–9 months of age, as well as synaptic loss and astro- and microgliosis, but neither neurofibrillary tangles nor appreciable neuronal loss. They differ from the APP_{Sw} and APP23 mice in which neurogenesis was studied previously, in that both of those lines lack the APP Indiana mutation. We report here that, compared to WT littermates, PDGF-$APP_{Sw,Ind}$ mice show increased neurogenesis in both SGZ and SVZ, consistent with findings in hippocampus of patients with AD, suggesting that enhancement of neurogenesis is an inherent feature of AD.

Materials and Methods

Mice. Experiments were conducted in accordance with National Institutes of Health guidelines and approved by local committee review. PDGF-$APP_{Sw,Ind}$ mice (19) were provided by Lennart

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Abbreviations: SVG, subventricular zone; DG, dentate gyrus; SGZ, subgranular zone; AD, Alzheimer's disease; DCX, doublecortin; APP, amyloid precursor protein; PS1, presenilin 1; PDGF, platelet-derived growth factor β.

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Mucke (Gladstone Institute of Neurological Disease, San Francisco) and used at 3 months and 1 year of age. Transgenic lines were maintained by heterozygous crosses with C57BL/6J breeders (Charles River Laboratories). Because familial AD caused by APP mutations is inherited in autosomal dominant fashion, the transgenic animals used were heterozygous with respect to the transgenes; nontransgenic littermates were used as controls. Hippocampal cellularity was evaluated by cresyl violet staining, and amyloid deposition was detected by immunohistochemistry, as described below, using mouse monoclonal anti-β-amyloid 3D6 (1:100), provided by Dennis Selkoe (Harvard Medical School, Boston).

BrdUrd Labeling and Cell Counting. BrdUrd (50 mg/kg, Sigma) was dissolved in saline and given i.p., twice daily at 8-h intervals, for 3 consecutive days, and mice were killed 1 week later. Brain sections were stained with mouse monoclonal anti-BrdUrd (Roche Molecular Biochemicals; 2 μg/ml) and biotinylated goat-anti-mouse IgG (Vector Laboratories, 1:200), and staining was visualized with diaminobenzidine and H_2O_2 as described in detail elsewhere (20). BrdUrd-positive cells in SGZ and SVZ were counted blindly in five to seven diaminobenzidine-stained, 50-μm coronal sections per animal, spaced 200 μm apart. Cells were counted under high power on a Nikon E800 microscope with a Magnifire digital camera, and the image was displayed on a computer monitor. Results were expressed as the average number of BrdUrd-positive cells per section.

Immunohistochemistry. Tissue was immersion-fixed in 4% paraformaldehyde in PBS (pH 7.5) and embedded in paraffin, and 6-μm sections were deparaffinized with xylene and rehydrated with ethanol. After blocking peroxidase activity with 1% H_2O_2, sections were incubated in blocking buffer (2% horse serum/0.2% Triton X-100/0.1% BSA in PBS) for 1 h at room temperature. In addition to the BrdUrd primary antibody described above, affinity-purified goat polyclonal anti-DCX (Santa Cruz Biotechnology, 1:200) was also used in single-label studies. Primary antibody was added in blocking buffer and incubated with sections at 4°C overnight. The secondary antibody was biotinylated goat anti-mouse or donkey anti-goat IgG (Vectastain Elite ABC, Vector Laboratories, 1:200). Sections were processed by using a Vector ABC kit. After two washes with PBS, sections were incubated for 10 min at room temperature with 0.5% biotinylated tyramine and 0.01% H_2O_2 in PBS, washed with PBS, and treated with ABC reagents. The horseradish peroxidase reaction was detected with diaminobenzidine and H_2O_2. Alternate sections were incubated without primary antibody as a control.

For double-label fluorescence immunohistochemistry, the primary antibodies were anti-BrdUrd and anti-DCX, as described above, as well as affinity-purified goat polyclonal anti-Neuro D (Santa Cruz Biotechnology, 1:100). The secondary antibodies were rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, 1:200). Fluorescence signals were detected with a Nikon E800 microscope at excitation/emission wavelengths of 535/565 nm (rhodamine, red) and 470/505 (FITC, green). Results were recorded with a Magnifire digital camera (ChipCoolers, Warwick, RI). Controls included omitting or preabsorbing primary or omitting secondary antibody. Selected images were viewed at high magnification by using a Nikon PCM-2000 laser-scanning confocal microscope, and SIMPLE PCI imaging software (Compix, Cranberry Township, PA) was used to confirm colocalization of markers.

Results

BrdUrd Labeling Is Increased in DG-SGZ of 3-Month-Old and 1-Year-Old PDGF-APP$_{Sw,Ind}$ Mice. The DG-SGZ is a major neuroproliferative region in the adult brain and a site of increased neurogenesis in AD (9), and the nearby Ammon's horn is one of the earliest and most severely affected brain regions in AD (21, 22). If injured tissue signals to neuroproliferative zones of the brain to stimulate neurogenesis, an early response in DG-SGZ might be expected for disorders, like AD, that prominently affect the hippocampus. Consistent with published findings (19), we detected amyloid deposition in 1-year-old, but not 3-month-old, PDGF-APP$_{Sw,Ind}$ mice and neuronal loss in neither (Fig. 1). Thus, for example, CA1 cell counts were no different in 1-year-old PDGF-APP$_{Sw,Ind}$, compared with WT mice ($P = 0.15, n = 3$ per group). In 3-month-old mice, the number of BrdUrd-labeled cells in DG-SGZ was increased ≈2-fold in PDGF-APP$_{Sw,Ind}$ mice, compared with WT littermates (Fig. 2 *A* and *B*). By 1 year, BrdUrd labeling in WT mice was reduced to ≈10% of levels measured at 3 months (Fig. 2 *C* and *D*), consistent with previous reports of an age-related decline in neurogenesis in DG-SGZ in WT animals (23). Again, however, the number of BrdUrd-labeled cells was roughly twice as high in DG-SGZ of PDGF-APP$_{Sw,Ind}$ mice as in WT mice.

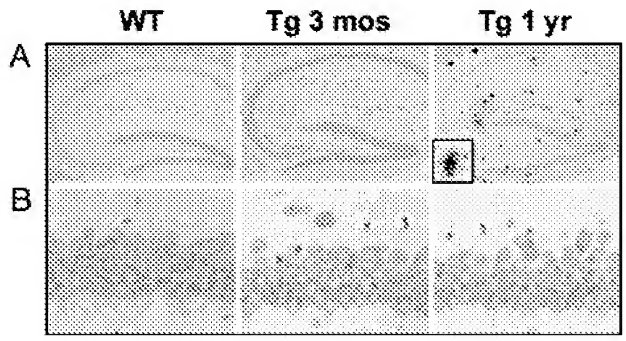

Fig. 1. Hippocampal histology in WT and PDGF-APP$_{Sw,Ind}$ transgenic (Tg) mice. β-Amyloid immunohistochemistry (*A*) and cresyl violet staining of CA1 (*B*) show amyloid deposition in 1-year-old but not 3-month-old transgenic mice, and no CA1 neuronal loss at either age.

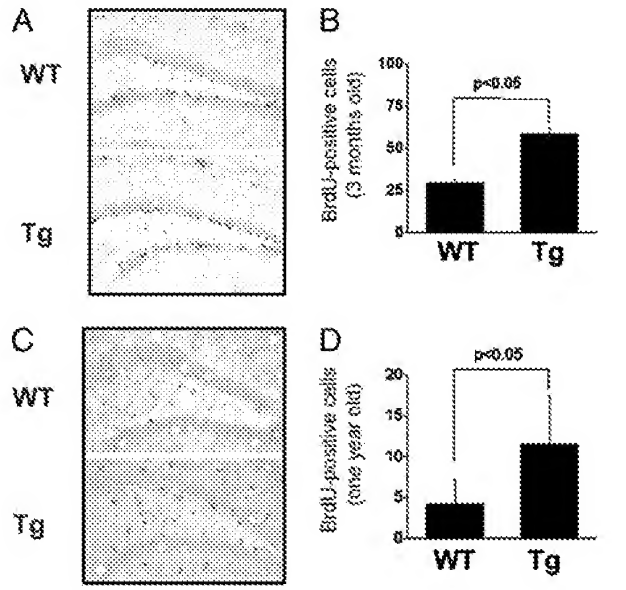

Fig. 2. BrdUrd labeling of cells in DG-SGZ of 3-month-old (*A* and *B*) and 1-year-old (*C* and *D*) WT and PDGF-APP$_{Sw,Ind}$ transgenic (Tg) mice. BrdUrd-labeled cells appear as black dots in *A* and *C*. The significance of differences between the number of BrdUrd-immunopositive cells in WT and transgenic mice in *B* and *D* was determined by Student's *t* test ($n = 3$–4).

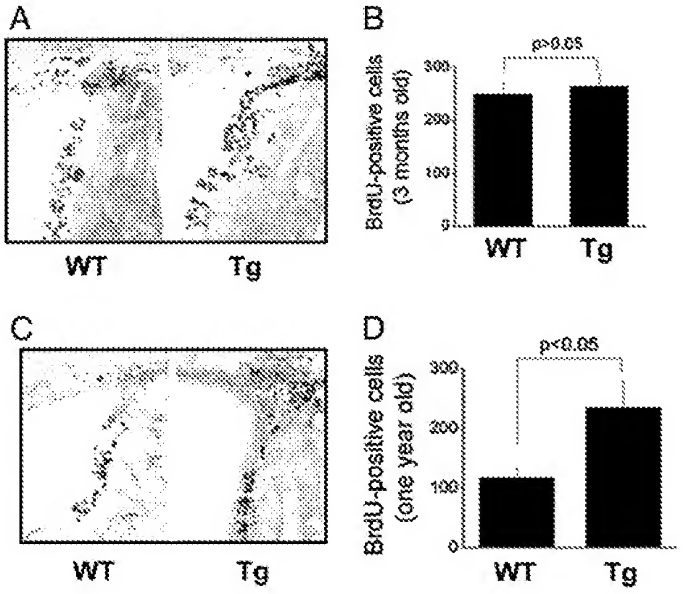

Fig. 3. BrdUrd labeling of cells in SVZ of 3-month-old (*A* and *B*) and 1-year-old (*C* and *D*) WT and PDGF-APP$_{Sw,Ind}$ transgenic (Tg) mice. BrdUrd-labeled cells appear as black dots in *A* and *C*. The significance of differences between the number of BrdUrd-immunopositive cells in WT and transgenic mice in *B* and *D* was determined by Student's *t* test (n = 3–4).

BrdUrd Labeling Is Increased in SVZ of 1-Year-Old, but Not 3-Month-Old, PDGF-APP$_{Sw,Ind}$ Mice. The effects of AD or animal models of AD on SVZ neurogenesis have not been reported, although the SVZ is a major location of injury-induced neurogenesis (2) and the site of origin for newborn neurons migrating to areas of injury (4, 6, 24) after stroke. Because the stroke models used involve occlusion of the middle cerebral artery, producing infarction in striatum and cerebral cortex, injury-induced SVZ neurogenesis seems to reflect damage to those regions. In contrast to our findings in DG-SGZ, 3-month-old PDGF-APP$_{Sw,Ind}$ mice showed no increase in BrdUrd labeling in SVZ, compared to WT littermates (Fig. 3*A* and *B*). However, at 1 year, when the number of BrdUrd-labeled cells in SVZ of WT mice had declined to ≈40% of levels measured at 3 months, BrdUrd labeling was ≈50% higher in PDGF-APP$_{Sw,Ind}$ mice than in WT mice (Fig. 3 *C* and *D*). The greater age-related decrease in neurogenesis in DG-SGZ than in SVZ of WT mice in this study is consistent with previous results in aging rats (20). The delayed enhancement of neurogenesis that we observed in SVZ, compared to DG-SGZ, may be related to the earlier and more pronounced hippocampal pathology in the PDGF-APP$_{Sw,Ind}$ model.

Expression of Immature Neuronal Markers Parallels BrdUrd Labeling in DG-SGZ and SVZ of PDGF-APP$_{Sw,Ind}$ Mice. Because BrdUrd labels the nuclei of replicating cells irrespective of cell lineage, additional markers are required to establish their phenotype. DCX is a microtubule-stabilizing factor that is expressed specifically in immature neurons, including those found in DG-SGZ and SVZ of adult brain (2, 25). DCX seems to promote the migration of these neurons because antisense knockdown of DCX expression inhibits migration (26). We have used DCX previously as a marker for newborn neurons in normal and injured brain, including the brains of patients with AD (9). To determine whether the increase in BrdUrd labeling in DG-SGZ and SVZ of PDGF-APP$_{Sw,Ind}$ mice is associated with an increase in the number of DCX-immunoreactive cells, we stained sections through DG-SGZ and SVZ of 3-month-old and 1-year-old WT and PDGF-APP$_{Sw,Ind}$ mice with an antibody against DCX.

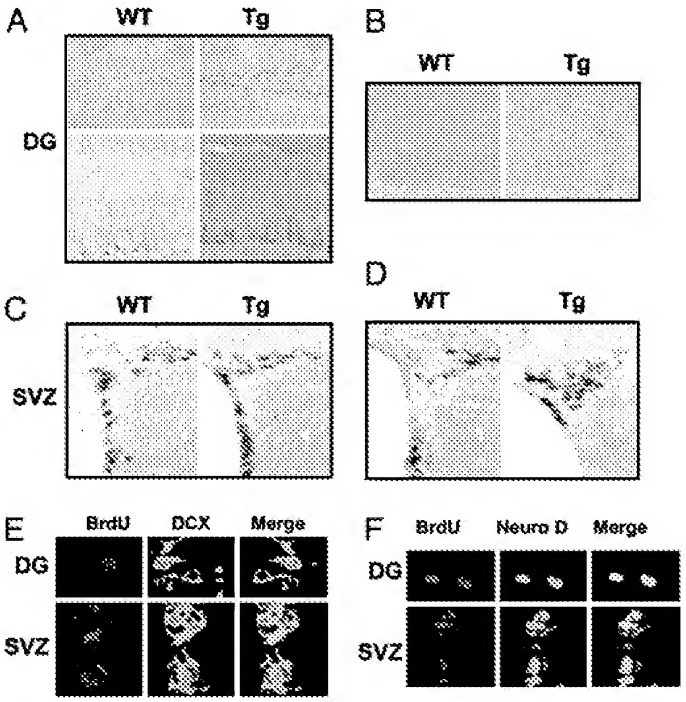

Fig. 4. Expression of immature neuronal marker proteins in DG-SGZ and SVZ of WT and PDGF-APP$_{Sw,Ind}$ transgenic (Tg) mice. Sections through DG (*A* and *B*) and SVZ (*C* and *D*) of 3-month-old (*A* and *C*) and 1-year-old (*B* and *D*) WT and PDGF-APP$_{Sw,Ind}$ transgenic mice were stained with an antibody against DCX (brown). Note that the number of DCX-immunoreactive cells in *B* (like the number of BrdUrd-labeled cells in Fig. 2*D*) was close to 0. Double-label immunohistochemistry with antibodies against BrdUrd and DCX (*E*) or BrdUrd and Neuro D (*F*) was performed on sections from 3-month-old transgenic mice and showed expression of DCX and Neuro D in BrdUrd-labeled cells in both DG-SGZ and SVZ.

Consistent with the results of BrdUrd-labeling experiments, DCX immunoreactive cells were increased in abundance in DG-SGZ of 3-month-old and 1-year-old mice, and in SVZ of 1-year- but not 3-month-old PDGF-APP$_{Sw,Ind}$ mice, compared with WT control mice (Fig. 4 *A* and *B*). Moreover, BrdUrd labeled the same cells in DG and SVZ that expressed DCX and a nuclear marker of immature neurons, the neurogenic differentiation factor Neuro D (Fig. 4 *C–F*).

Discussion

The main finding of this study is that in PDGF-APP$_{Sw,Ind}$ transgenic mice, as in the brains of patients with AD, the generation of new neurons is increased, compared with basal levels of neurogenesis observed in controls. Several conclusions emerge from this finding.

First, because it can be recapitulated in an animal model, the enhancement of neurogenesis observed previously in the hippocampus of AD patients is likely to be a feature of the disease *per se*, and not an epiphenomenon related to, for example, nutritional state or medications. This is important because nutritional deficiency is common late in the course of AD, and neurogenesis is increased in anorexic (*anx/anx*) mutant mice (27), which die of starvation. In addition, several drugs that are used commonly by AD patients may also stimulate neurogenesis. These include acetylcholinesterase inhibitors like donepezil, because muscarinic cholinergic receptors are expressed on neuronal progenitors and are coupled to cell proliferation (28); memantine, because other *N*-methyl-D-aspartate receptor antagonists enhance neurogenesis (29); statins, which have been shown to increase neurogenesis (30); and nonsteroidal antiin-

flammatory drugs, considering that neurogenesis may be reduced by inflammation (31).

Second, neuronal loss is not required for injury-induced neurogenesis, which seems responsive to more subtle pathological triggers. The mice we studied, like all transgenic models of AD, exhibit some but not all features of the human disease. PDGF-APP$_{Sw,Ind}$ mice show early (2–4 months) defects in synaptic transmission, before β-amyloid deposits, neuronal loss, and gliosis are seen, and neurofibrillary tangles do not occur in these mice (19). This finding suggests that synaptic abnormalities, which seem to involve a decrease in the number of functional synapses in the Schaffer collateral projection from CA3 to CA1 hippocampal neurons (19), may be the stimulus for neurogenesis. That injury-induced neurogenesis can occur without neuronal loss may seem counterintuitive, especially if neurogenesis is seen as a mechanism for cell replacement. However, other neurological disorders, like seizures (1), stimulate neurogenesis without necessarily killing neurons, and neurogenesis-promoting growth factors (or other mediators) released by pathological processes associated with cell death, such as hypoxia or ischemia (32), could also be released in response to lesser degrees of injury.

Third, although enhancement of neurogenesis in AD transgenic mice does not seem to depend on β-amyloid deposition or neuronal loss, it does seem to reflect the spatiotemporal pattern of disease pathology. Thus, BrdUrd labeling was increased at 3 months in DG-SGZ, but not until 1 year in SVZ, perhaps because of the proximity or connectivity of DG-SGZ to the earliest sites of AD pathology. These include the entorhinal cortex, which projects via the perforant and alvear pathways to the hippocampus, and lesions of which stimulate DG-SGZ neurogenesis (29), and the CA1 sector of the hippocampus, which also bears the brunt of injury from global ischemia, in which neurogenesis is likewise increased (33). The magnitude of transgene-induced increase in BrdUrd labeling was similar (≈2-fold over control) in 3-month-old DG-SGZ and in 1-year-old DG-SGZ and SVZ, indicating that both regions had equivalent capacity for injury-induced neuroproliferation. We have observed larger fold increases in BrdUrd labeling after ischemia (2) and in the expression of immature neuronal markers in the brains of AD patients (9), suggesting that the magnitude of increase in neurogenesis may be related to the acuteness and duration of the inciting pathology. The fact that the full capacity for neurogenesis seen, for example, after ischemia, was not mobilized in our transgenic mice indicates that therapeutic enhancement of neurogenesis, beyond that induced by AD alone, may be possible. This finding is consistent with the finding that growth factor administration can increase neurogenesis as much as 5-fold in the normal aged rat brain (20) and can potentiate neurogenesis induced by cerebral ischemia (34).

Our results contrast with prior findings from transgenic models of AD, in which mice expressing mutant forms of APP (13, 14, 17) showed impaired, rather than increased, neurogenesis. These disparities raise the possibility that different forms of AD may differ with respect to their association with neurogenesis, especially considering that sporadic AD is thought to account for the great majority of cases and is likely a heterogeneous disorder. One factor that distinguishes the transgenic mice we used from those used in prior studies of neurogenesis is the presence of the APP Indiana (V717F) mutation in our mice. Transgenic mice expressing this mutation alone show defects in synaptic transmission that are similar in nature to, but smaller in magnitude than, those seen in PDGF-APP$_{Sw,Ind}$ mice (19). It is of interest that the Schaffer collateral pathway affected in these mice and the perforant pathway that projects from entorhinal cortex to hippocampus and is affected early in AD both employ glutamate as a transmitter (35), and that interruption of glutamate neurotransmission is known to stimulate adult neurogenesis (36). This might suggest that a defect in glutamatergic transmission is what triggers neurogenesis in PDGF-APP$_{Sw,Ind}$ mice and patients with AD. Additional studies will be required to test this and alternative hypotheses.

The significance of neurogenesis in the adult human brain under physiological or pathological conditions is unknown. However, new functional neurons have been shown to arise from the adult human hippocampus (7). Moreover, depleting neuronal precursors in DG-SGZ with methylazoxymethanol acetate impairs hippocampal-dependent memory formation in rats (37), and ablation of these cells by cranial x-irradiation interferes with functional recovery from global cerebral ischemia in gerbils (38). These observations suggest that nascent neurons in the adult human brain might also have a functional role. As a corollary, the increased neurogenesis seen in diseases like AD may represent an endogenous brain-repair mechanism, the further stimulation of which could have therapeutic potential. The availability of an animal model of AD-induced neurogenesis should facilitate investigation of these issues.

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